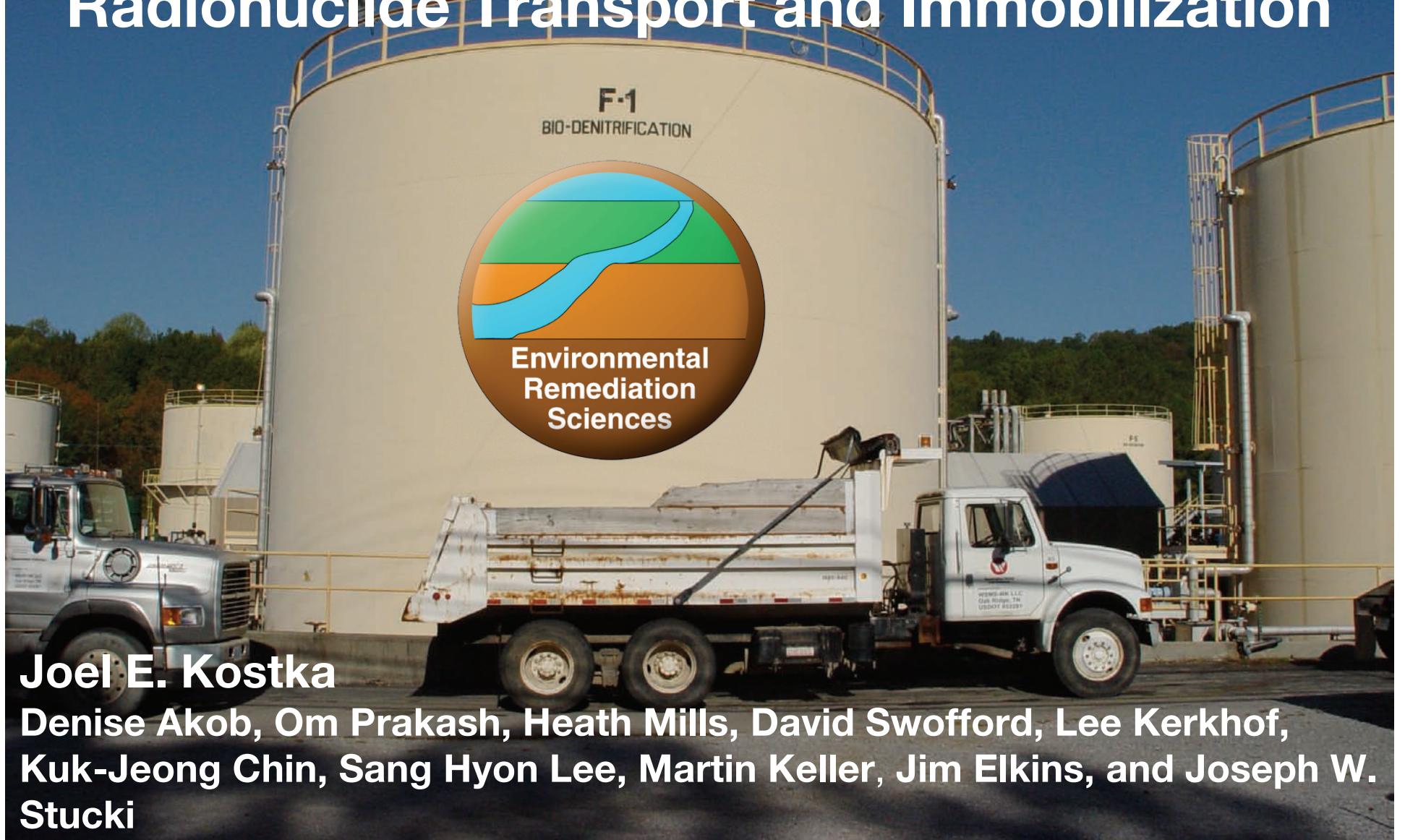


# Structure and Function of Subsurface Microbial Communities Affecting Radionuclide Transport and Immobilization



**Joel E. Kostka**

Denise Akob, Om Prakash, Heath Mills, David Swofford, Lee Kerkhof,  
Kuk-Jeong Chin, Sang Hyon Lee, Martin Keller, Jim Elkins, and Joseph W.  
**Stucki**

# Current Remediation Strategy

- Source zone treatment (including secondary sources)
  - biostimulation
- Natural attenuation and monitoring

✓ ERSD/ BER Long Term Performance

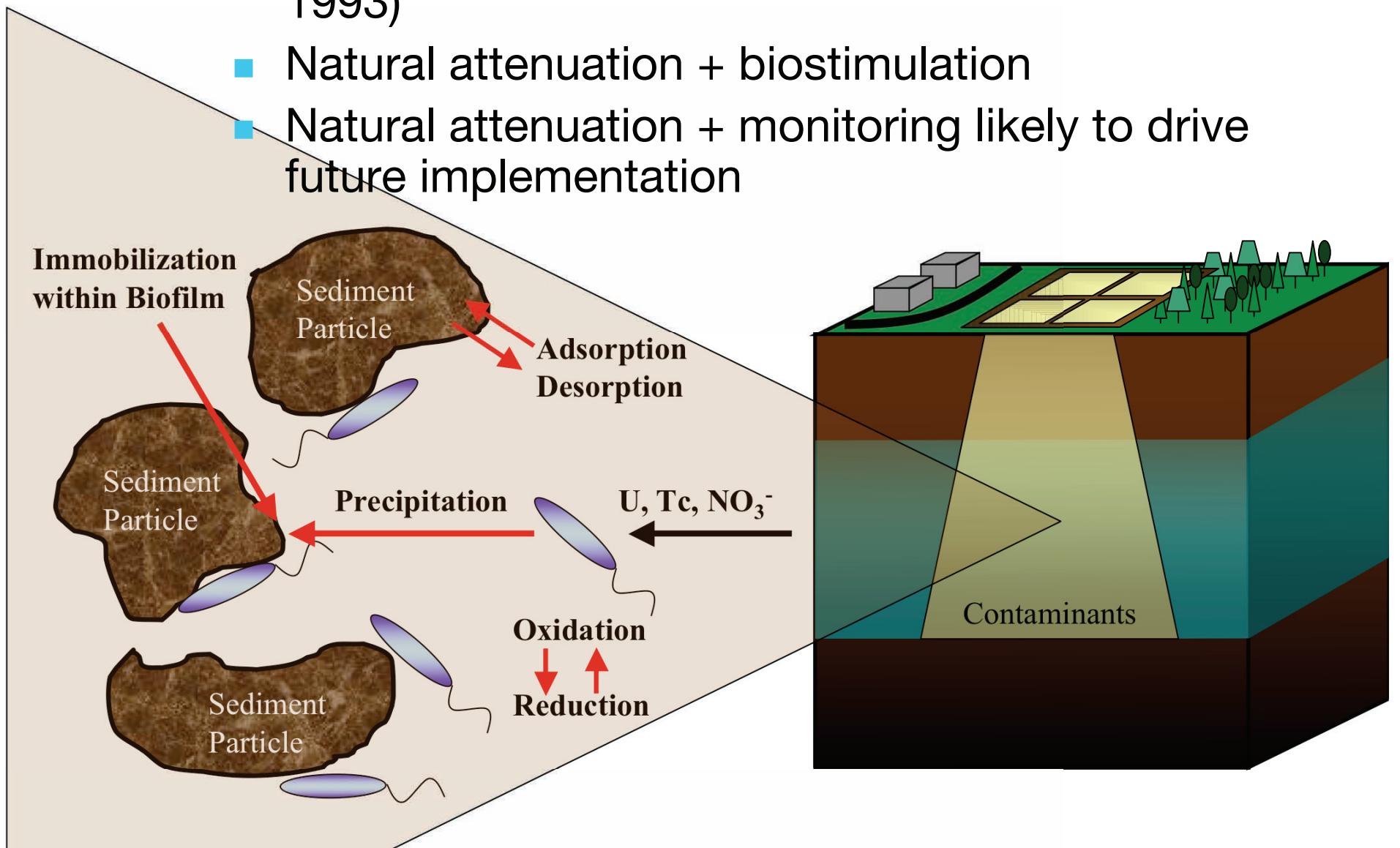
**Measure:** “provide (by 2015) sufficient scientific understanding to allow a significant fraction of DOE sites to incorporate coupled biological, chemical, and physical processes into decision making for environmental remediation”



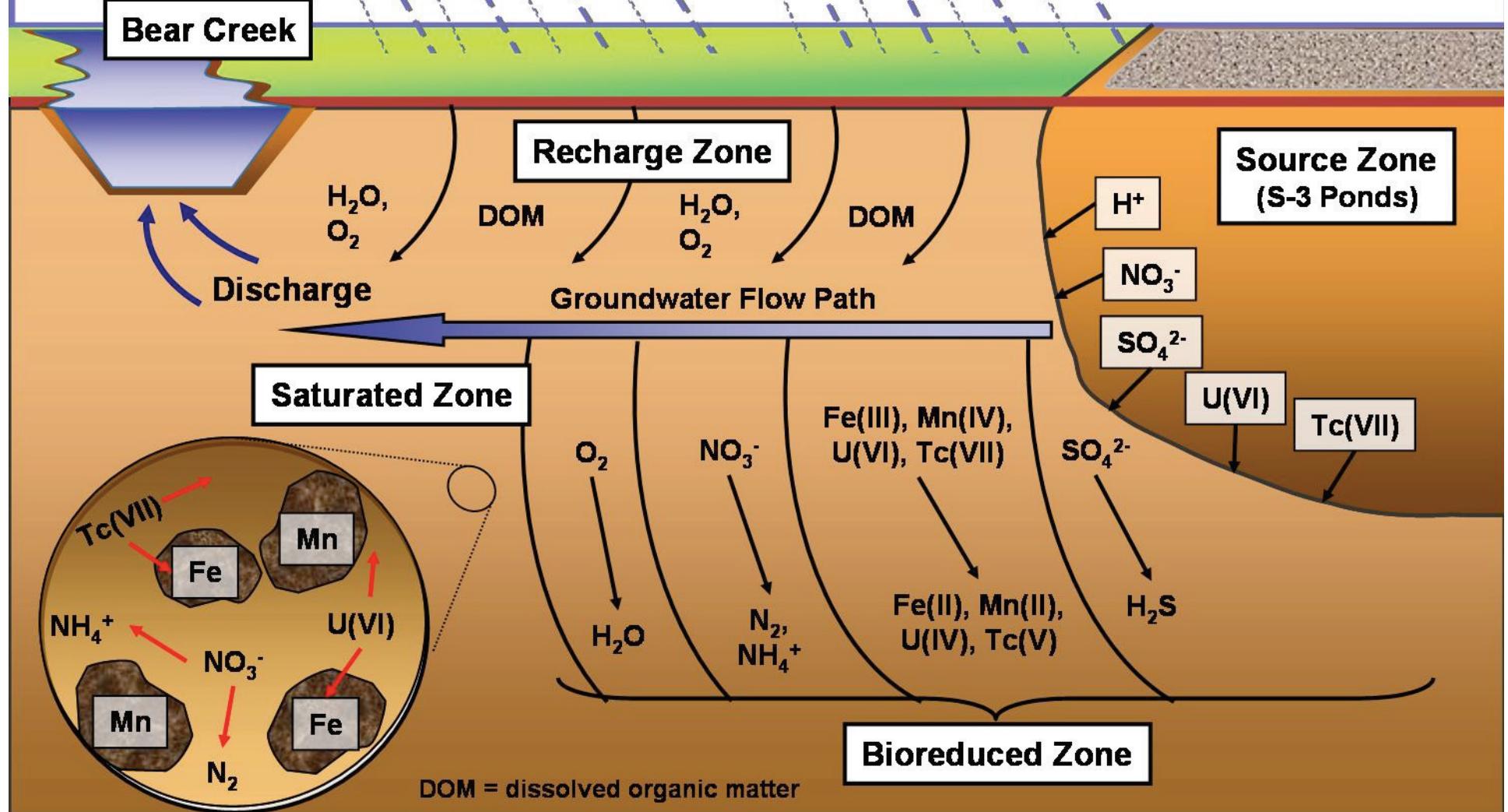
# Objectives

- 1) Isolate and characterize novel anaerobic metal- and nitrate-reducing prokaryotes from subsurface environments exposed to high levels of mixed contaminants (U(VI), nitrate, sulfate).
- 2) Elucidate the diversity and distribution of metabolically active metal- and nitrate-reducing prokaryotes in subsurface sediments.
- 3) Determine the biotic and abiotic mechanisms linking electron transport processes (nitrate, Fe(III), and sulfate reduction) to radionuclide reduction and immobilization.

- PROBLEM STATEMENT: Bioremediation potential dictated by the physiological requirements for the growth and metabolism of microorganisms (Tiedje, 1993)
- Natural attenuation + biostimulation
- Natural attenuation + monitoring likely to drive future implementation

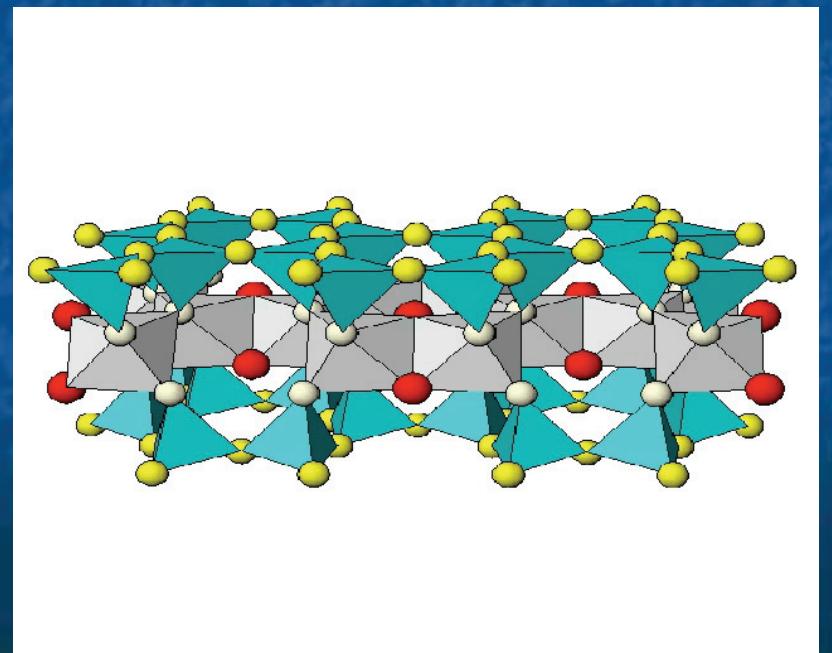


Subsurface  
microbiology and  
biogeochemistry  
at the watershed  
scale



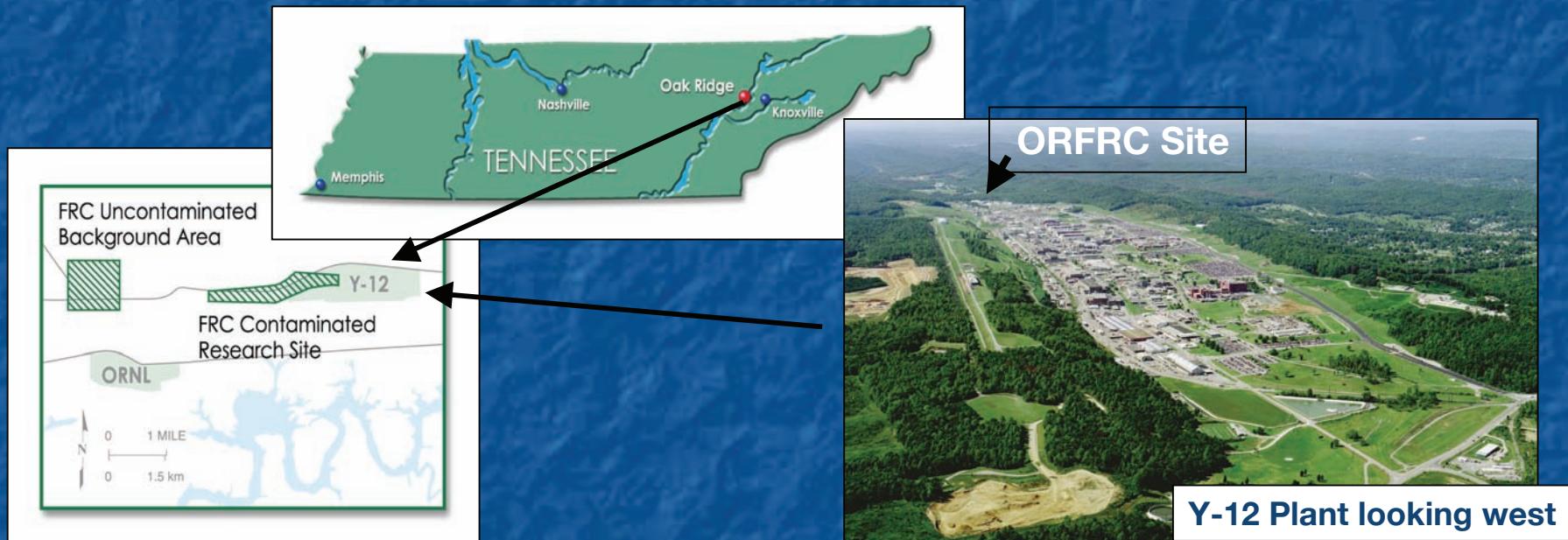
# U(VI) sorption/ precipitation with Fe minerals

- Fe minerals shown to be effective sorbents of U(VI) in subsurface
- U may be incorporated into the structure of Fe minerals through coprecipitation
- Sorption may inhibit U(VI) reduction
- Reduced Fe may catalyze abiotic U(VI)
- Clay minerals were recently shown to comprise half or more of the total Fe in terrestrial subsurface sediments
- Few studies have examined the role of clays in U(VI) sorption



# Field Site

## Oak Ridge Field Research Center (ORFRC)



# Site Description

The Oak Ridge IFC is located in eastern Tennessee and contains contaminated and uncontaminated field facilities as well as on-site and off-site laboratory facilities.

At the contaminated site, unlined surface impoundments received **acidic nitrate- and U-bearing waste** from 1951 to 1983 at a rate of 2.5 million gallons/year. Attempts were made to neutralize the waste and the waste trenches were capped as a parking lot in 1988.

The region receives ~1400 mm rainfall / y with 10% contributing to groundwater recharge and 40% contributing to surface water recharge.

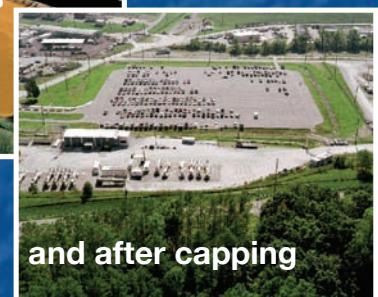
The subsurface media consist of **fractured saprolite weathered from interbedded shale and limestone and is conducive to rapid preferential flow of water and solutes.** The matrix porosity serves as a **"secondary contaminant source"** whose aerial extent is massive (tens of kilometers).

Both aqueous and solid phase geochemistry and microbiology are spatially and temporally diverse

Conceptual models have been developed for secondary sources, shallow and deep plumes, low and high pH saprolite plumes, and high pH gravel plumes.



S-3 Ponds during denitrification



and after capping

ORNL media consisting of interbedded fractured weathered shales and limestone

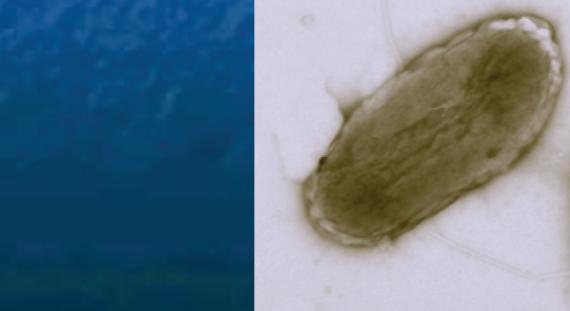
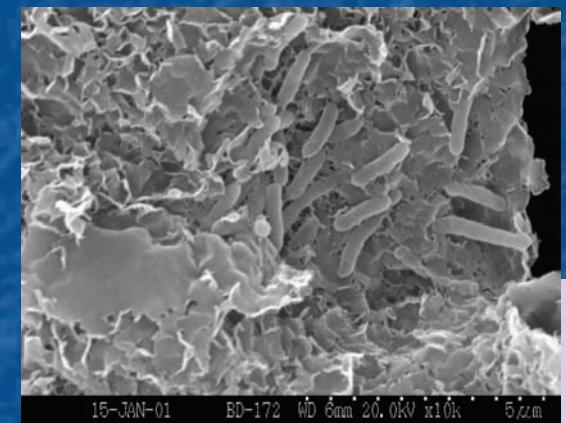
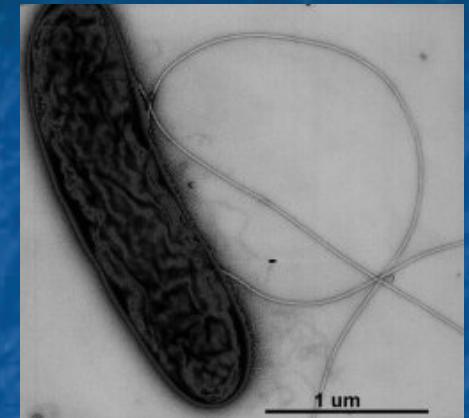


Saprolite



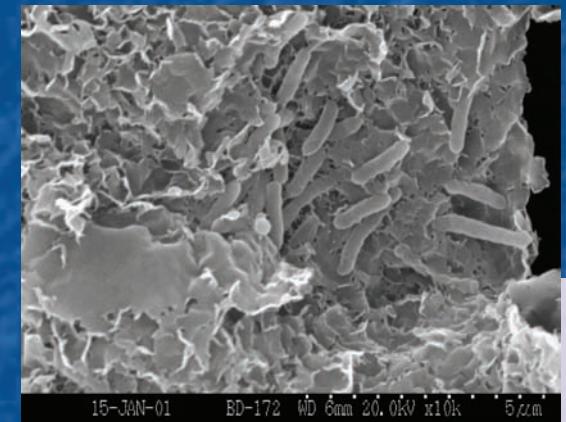
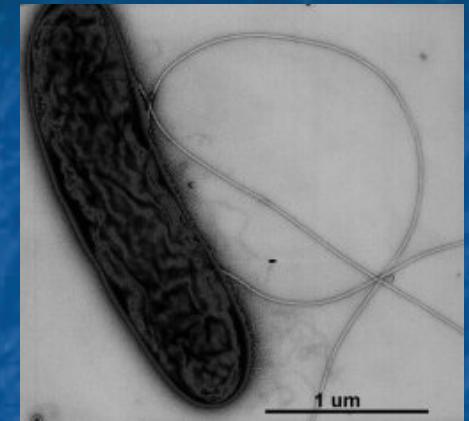
# Target Microbial Groups

- Nitrate-reducing prokaryotes
  - Denitrifiers
  - Anammox bacteria
  - Dissimilatory nitrate reduction to ammonium
- Metal-reducing prokaryotes
  - Fe(III)-reducers
  - Sulfate-reducers
  - Fermentative organisms
  - Consider shuttle mediated metal transformation



# Sulfate-reducing prokaryotes (SRP) vs. Fe(III)- reducing prokaryotes (FeRP)

- Broad phylogenetic groups
  - Deltaproteobacteria important to ecology of both
- Broad metabolic diversity
  - Heterotrophs, autotrophs, syntrophs
  - FeRP have broader respiratory metabolism-Shewanella group
- Evidence for overlap in metabolism
- FeRP must access solid electron acceptor
- Radiotracer assay and good functional gene target for SRP
- Fe(III) reduction rates difficult and tedious to measure
- dsr analysis has shown that SRP diversity much larger than previously thought



# Research Highlights

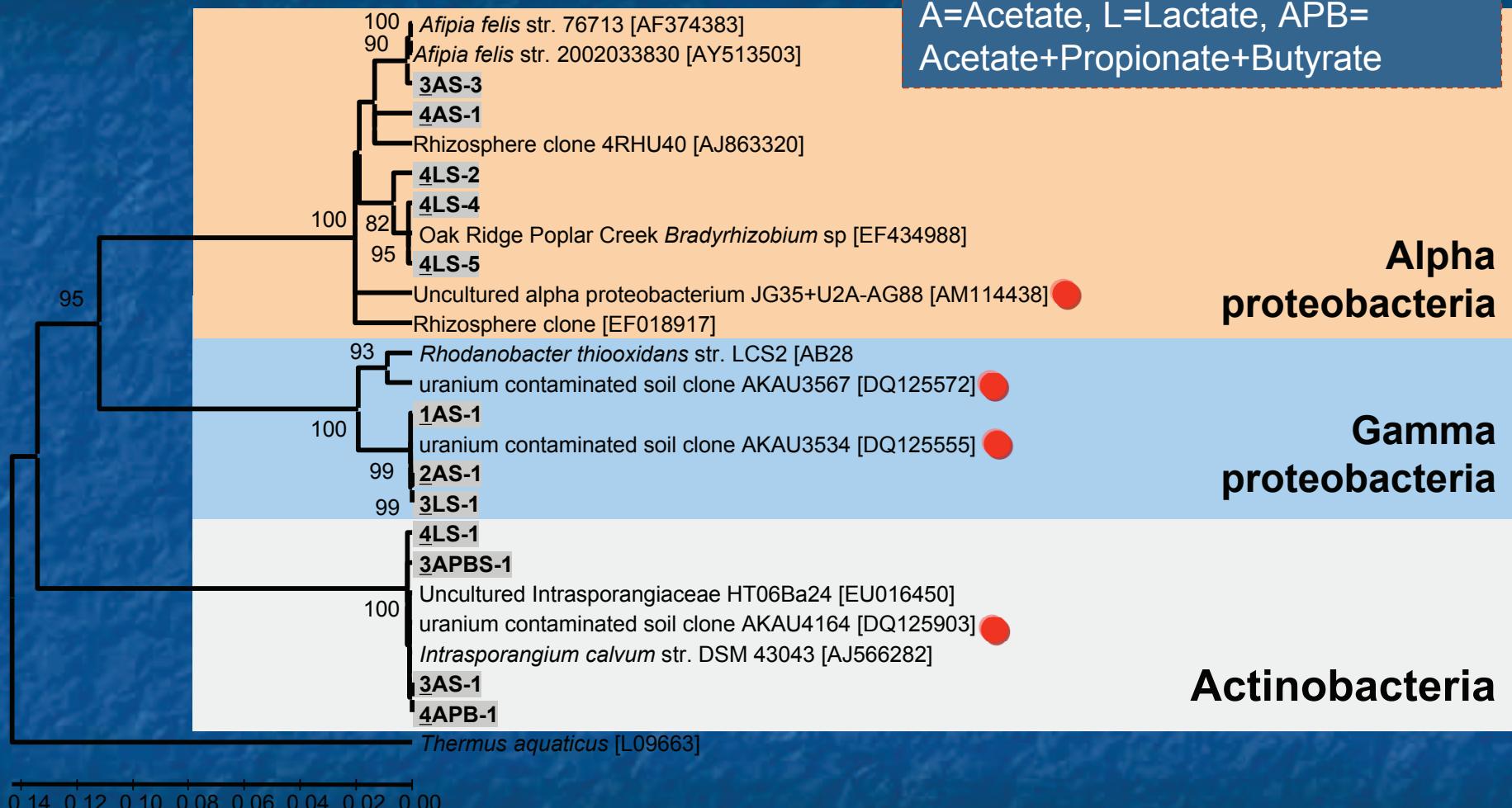
- Isolation and characterization of novel anaerobes to link molecular biology tools and physiology

# Cultivation of nitrate-respiring prokaryotes

- FB 107, Area 2, uranium-contaminated ORFRC
- Series of 20 enrichments in synthetic groundwater medium
- Systematic variation of electron acceptor and donor
- Treatments (at 0.5, 1, 10, 25 mM nitrate):
  - 10 mM acetate/ propionate/butyrate
  - 10 mM acetate
  - 10 mM lactate
  - 10 mM ethanol
  - no carbon substrate added
- Transferred to solid media
- Streaked for purity 3X
- Screened in liquid culture for growth on nitrate and nitrate reduction
- 12 pure cultures to date



# Phylogenetic analysis of partial 16S rRNA gene sequences recovered from nitrate-respiring bacterial isolates



Linearized neighbor-joining phylogenetic tree of Oak Ridge, TN Area 2 denitrifier isolate 16S rRNA gene sequences. A boot-strapped neighbor-joining tree was generated with 1000 tree reiterations. The scale bar represents from 0-14% sequence divergence. Isolate sequences are highlighted in gray. Red circles indicate sequences from uranium-contaminated sediments.

# Summary of Isolate Characteristics

- 12 pure cultures of nitrate-respiring bacteria from ORFRC subsurface sediments
  - 4 - Alphaproteobacteria > **Bradyrhizobiaceae** > **Afipia-like**
  - 4 - Actinobacteria > **Intrasporangiaceae** > **Intrasporangium-like**
  - 4 - Gammaproteobacteria > **Xanthomonadaceae** > **Rhodanobacter-like**
- All show high sequence identity to phylotypes retrieved from ORFRC subsurface and other U(VI)-contaminated environments
- Affiliated with organisms known to utilize nitrate and sulfur compounds, degrade aromatic hydrocarbons
- Rhodanobacter-like isolates
  - Closely related to clones from Brodie et al. (2006), abundant in metagenome sequences from acidic ORFRC groundwaters (Zhou et al.)
  - 3 species in genus; all isolated from soils
  - One strain isolated from sulfur particles of an anaerobic sludge reactor used in autotrophic denitrification process

# High Throughput Cultivation (HTC) of Novel Microbes from ORFRC Contaminated Groundwater

- Improve fluorescence-activated cell sorting (FACS)-based methods for individual microbe capture and cultivation
- Develop microfluidic architectures for highly parallel capture, cultivation, and release
- Develop methods for down-stream capture of released microbes for whole-genome amplification
- Use of the microfluidic system to cultivate microorganisms from ORFRC site and to search for novel nitrate, sulfate, and metal reducing microorganisms.



✓ **Jim Elkins and Martin Keller, ORNL**

# Research Highlights

- Determine the mechanisms linking electron transport processes to radionuclide reduction and immobilization

# Rationale for quantification of gene expression

- Desired target = gene that is highly conserved in phylogenetically distinct group and for which expression patterns are correlated to metabolic rates
- SRP
  - *dsrA* highly conserved amongst the SRP
- FeRP
  - *gltA* highly conserved amongst Geobacteraceae
  - Geobacteraceae often predominate in Fe(III)-reducing zones
  - Citrate synthase (*gltA*)- key gene in the incorporation of acetate into the TCA cycle
  - *gltA* present in all Geobacteraceae members examined, more closely related to that of eukaryotes than other prokaryotes

# Microcosm experiment - objectives

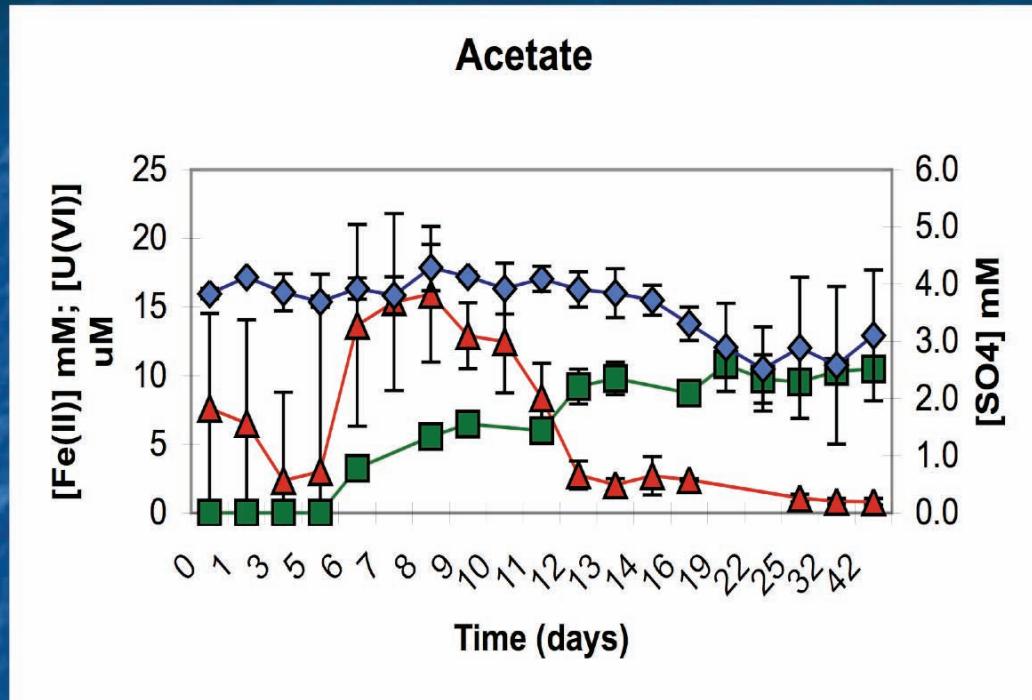
- Objectives:
  - To identify and quantify the metabolically active SRP and FeRP that catalyze U(VI) reduction
  - To determine which electron donor promotes U(VI) reduction by these groups

# Microcosm experiment - methods

- Area 2 sediment with artificial groundwater
- Treatments: 5 mM carbon substrates, 2.5 mM sulfate
- Quantify rates of electron acceptor and donor utilization
- Molecular methods
  - mRNA extraction; RT-real-time PCR
    - Citrate synthase, gltA
    - Dissimilatory sulfite reductase, dsrA
  - Stable isotope probing with  $^{13}\text{C}$  labeled substrates

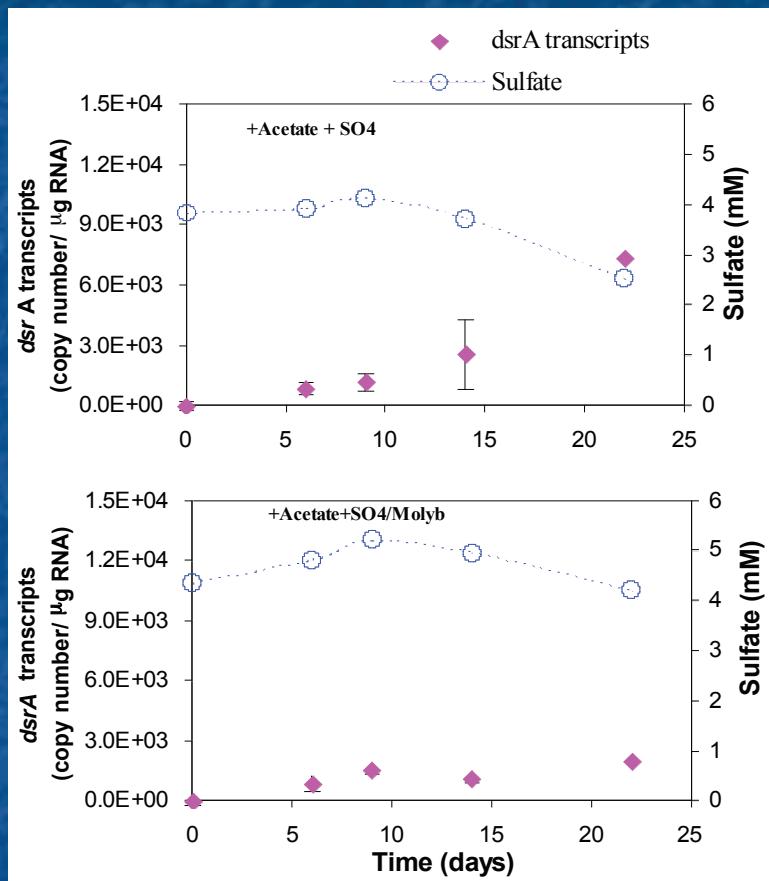


# Electron acceptor and donor utilization

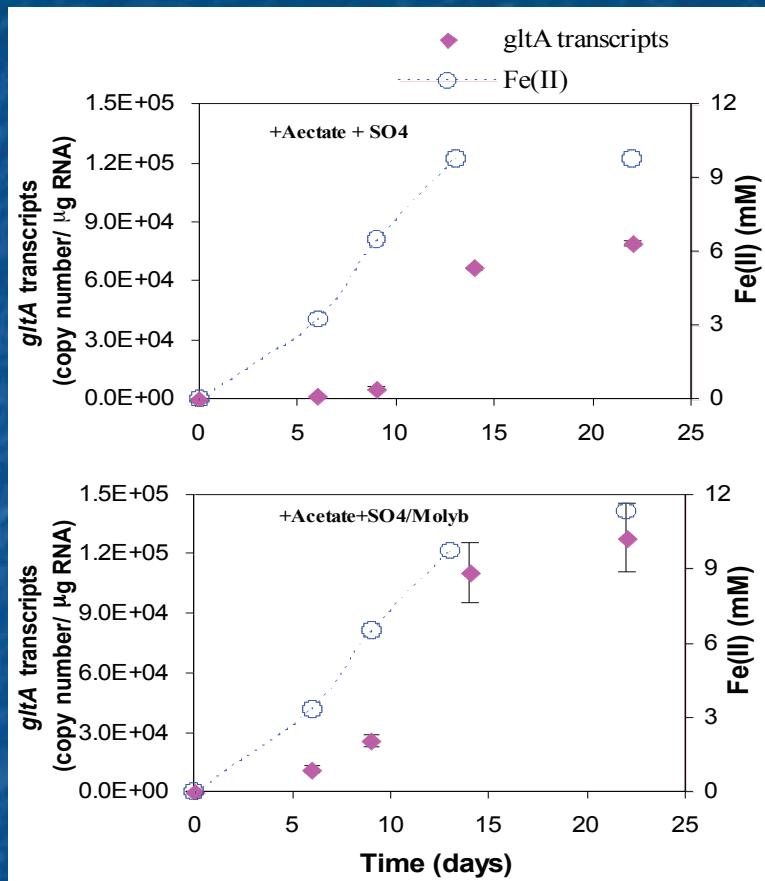


# Expression of *dsrA* gene & *gltA* gene during sulfate & Fe(III) reduction in ORFRC acetate-amended microcosm sediments

(A) *dsrA*



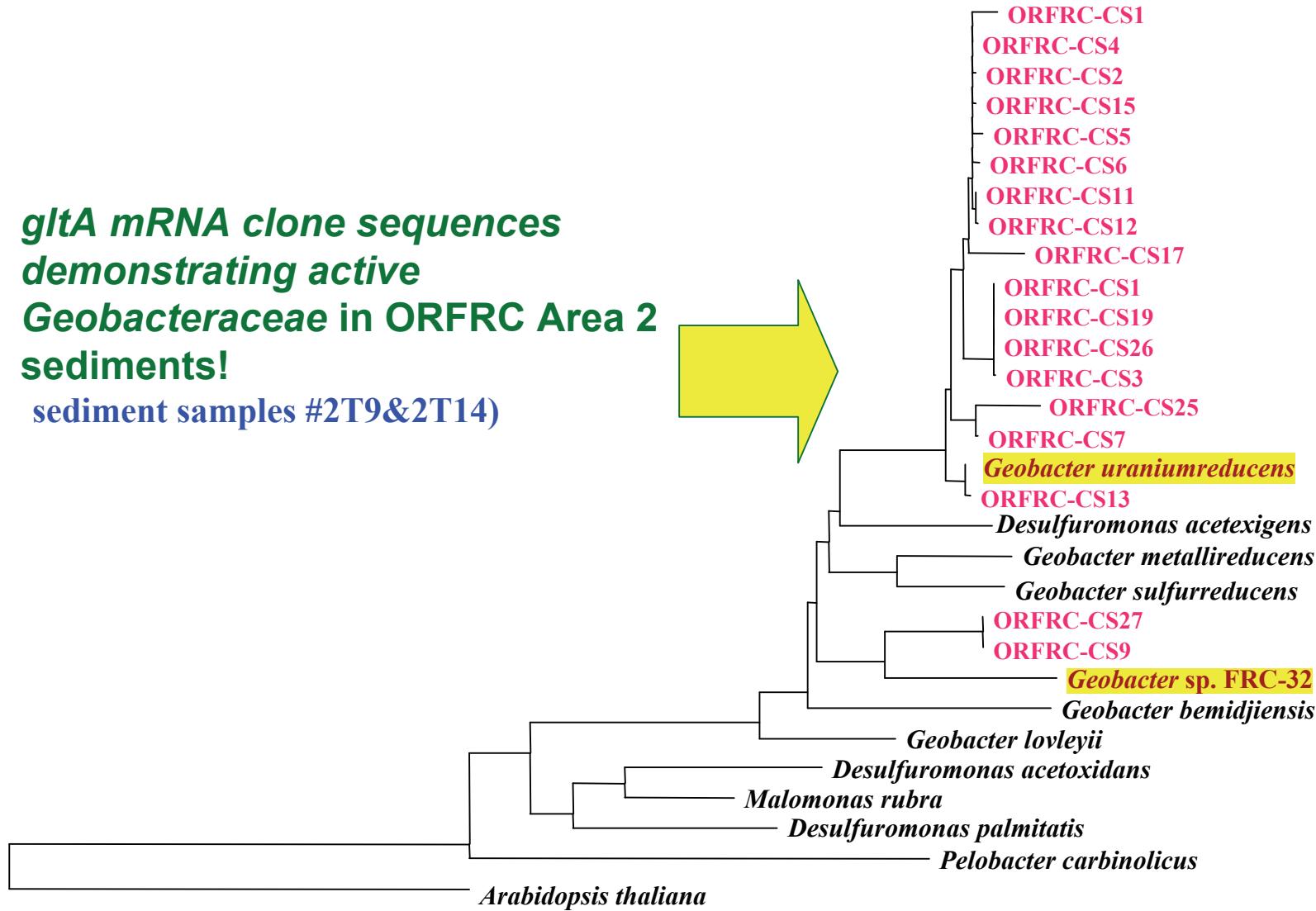
(B) *gltA*



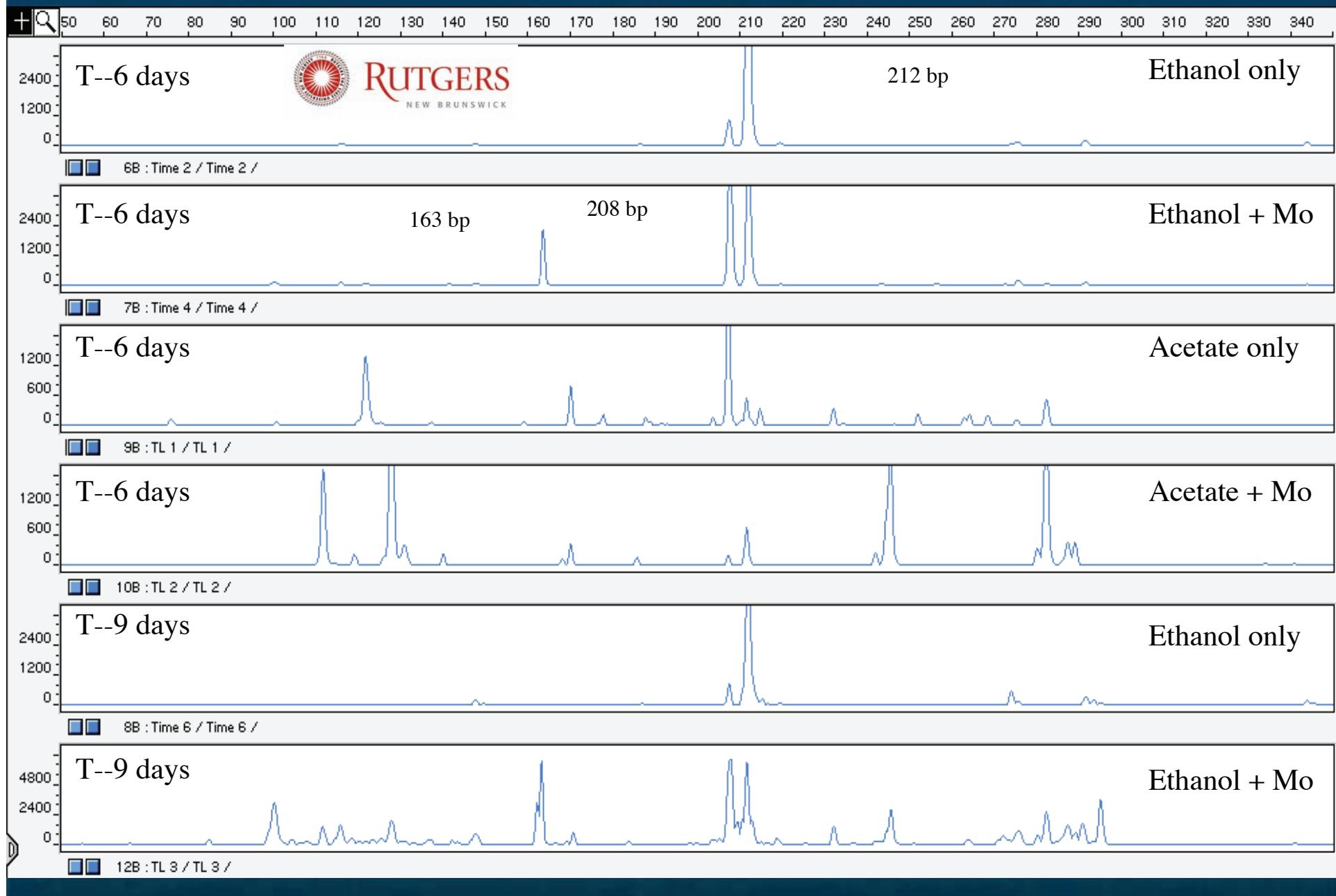
**(A) *dsrA* gene expression and sulfate concentration in ORFRC Area 2 SIP microcosm sediments incubated with <sup>13</sup>C-acetate + sulfate or <sup>13</sup>C-acetate + sulfate + molybdate amendment. (B) *gltA* gene expression and Fe(II) concentration in ORFRC Area 2 SIP- microcosm sediments incubated with <sup>13</sup>C-acetate+ sulfate or <sup>13</sup>C-acetate + sulfate + molybdate amendment.**

## Phylogenetic tree indicating the relationship of selected citrate synthase gene (*gltA*) mRNA clone sequences retrieved from ORFRC Area 2 sediment microcosm

*gltA* mRNA clone sequences  
demonstrating active  
**Geobacteraceae** in ORFRC Area 2  
sediments!  
sediment samples #2T9&2T14)



# Ethanol and acetate microcosm treatments with/without molybdate



# Conclusions from stable isotope probing:

- The 212 bp peak is the dominant TRF in the ethanol only microcosms and is taking up the bulk of the labeled carbon.
- The 212 bp peak appears inhibited by molybdate, but is not completely inactive.
- More active TRF's are seen in the molybdate addition studies than in the electron donor alone.
- The 163 bp TRF is only active in the presence of ethanol and Mo.
- The 112, 128, 245, and 282 bp peaks are active in the presence of Mo and ethanol or Mo and acetate.



# Fe Mineralogy of Area 2 Microcosm Sediments

## Methods:

**Fe form and speciation characterized using cryostated Mossbauer spectroscopy**  
**All spectra were recorded with the sample at liquid He temperature (4.2 to 6 K)**

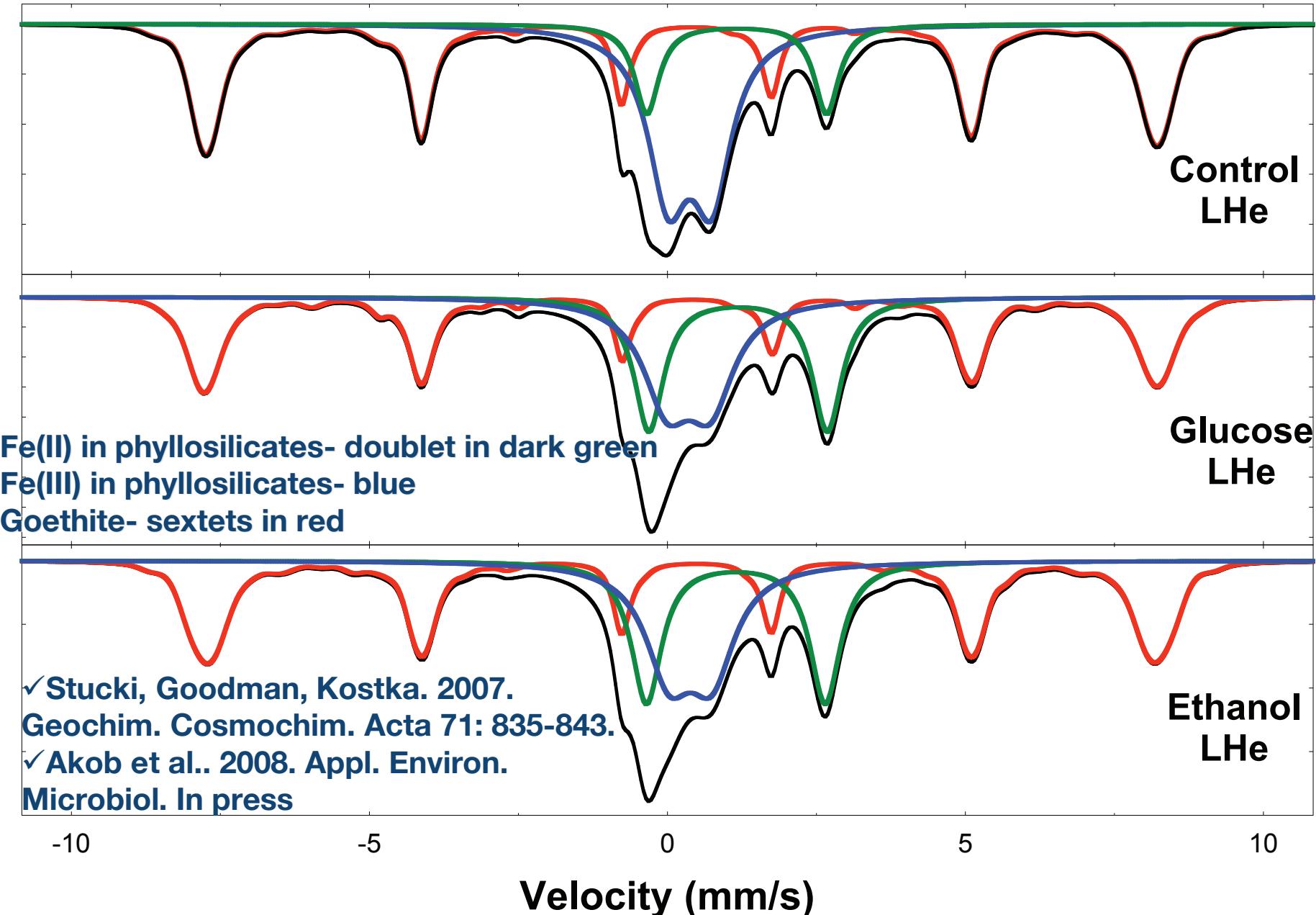
Treatment	Fe State or Phase	Relative Area %
Control	Fe(III) in Silicate	39
	Fe(II) in Silicate	13
	Goethite	48
Glucose	Fe(III) in Silicate	31
	Fe(II) in Silicate	26
	Goethite	43
Ethanol	Fe(III) in Silicate	29
	Fe(II) in Silicate	26
	Goethite	45

**Fe(III) bound in phyllosilicate clay minerals dominates the bioreducible Fe**

**Clay mineral reduction directly linked to acetate consumption and increase in Geobacteraceae gltA expression level**

**Akob et al.. 2008. Appl. Environ. Microbiol. In press**

✓ Joe Stucki, University of Illinois



# **Benefit to the U.S. DOE**

- Molecular proxies for process monitoring
- Establish cause and effect relationships between contaminant removal and the activity of specific microbial groups under in situ conditions
- New model organisms to link genetics and physiology for key microbial groups
- Determine impact of inhibitors (cocontaminants) and undesirable metabolisms on contaminant remediation processes
- Link new knowledge of microbial function to kinetics and constraints of enzymatic processes
- Determine biomass and turnover of specific microbial groups for input into site models

# Conclusions

- New nitrate-respiring bacteria were isolated from ORFRC subsurface sediments that will enable us to link the molecular detection and the physiology of this important group. All isolates show high sequence identity to phylotypes retrieved from U(VI)-contaminated environments as well as organisms known to utilize nitrate and sulfur compounds, degrade aromatic hydrocarbons.
- Molecular proxies for process monitoring were demonstrated in Area 2 subsurface sediments. Expression of dsrA and gltA tracked with sulfate reduction and iron reduction activity, respectively. However, other factors such as availability of electron acceptors may impact gene expression
- Expression of genes for central metabolism may be more closely linked to rates than genes for respiration

# Conclusions

- Phylogenetic analysis of *gltA* mRNA clone sequences demonstrated that groups closely related to *Geobacter uraniumreducens* and *Geobacter FRC-32* are active and abundant in ORFRC Area 2 microcosm sediments.
- Phylogenetic analysis of SRB-specific 16S rRNA clone sequences indicated that groups closely related to family *Desulfobacteraceae* are active and abundant in ORFRC Area 2 field sediments.
- The conventional ferrozine method is unreliable for giving total Fe(II) or total Fe in sediments because:
  - HCl is the wrong acid to dissolve silicates.
  - Fe(III)-ferrozine complexes are photochemically reduced to Fe(II)-ferrozine
- Clay minerals account for a large fraction of Fe(III) minerals available for bioreduction in subsurface sediments

# Current/ Future

- Expand all tasks into highly contaminated source zone
- Screen and characterize new nitrate-respiring isolates
- Use microfluidic system to search for novel nitrate, sulfate, and metal reducing microorganisms from ORFRC site
- Phylogenetic analysis of large cDNA fragments to confirm identification of active FeRP and SRP
- To relate to population abundance, quantify transcripts per cell by normalizing to gene copy number of dsr and 16S
- Strengthen databases for process-specific genes (nosZ, nirS, gltA, dsr)
- Nitrate reduction with bioreduced clay minerals
- Link bioreduction of Fe(III) minerals to changes in cation exchange capacity and fate of uranium

# Posters - All on Tuesday

- Kostka et al. - Structure and Function of Subsurface Microbial Communities Affecting Radionuclide Transport and Bioimmobilization
- Akob et al. - Targeting the Metabolically Active Iron(III)- and Sulfate-Reducing Bacteria with a High Potential for U(VI) Bioimmobilization in Contaminated Subsurface Sediments

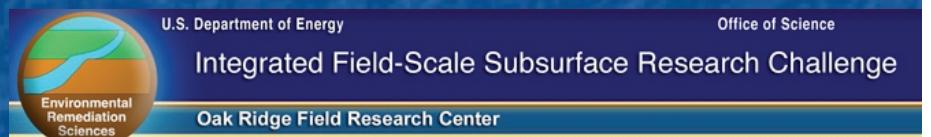
# 108th General ASM Meeting, Boston, MA

## The Shallow Subsurface Biosphere: Critical Geobiological Interface and Window into the Deep Biosphere

- Thursday, June 5th, 8 am
- Coconvenors: Joel E. Kostka and Bo Barker Joergensen
- Goal: to bring together microbiologists working in the shallow terrestrial and marine subsurface
- Thanks to Patty Sobecky and Lee Kerkhof, leaders of the Microbial Ecology Division, for their support!

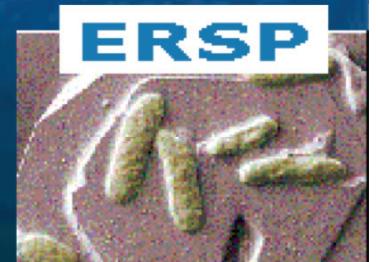
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